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Placental immune response to apple allergen in allergic mothers

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ABSTRACT

The immunological milieu in the placenta may be crucial for priming the developing foetal immune system. Early imbalances may promote the establishment of immune-mediated diseases in later life, including allergies. The initial exposure to allergens seems to occur in utero, but little is known about allergen-induced placental cytokine and chemokine release. The release of several cytokines and chemokines from placenta tissue after exposure to mast cell degranulator compound 48/80 or apple allergen in placentas from allergic and healthy mothers was to be analysed. Four placentas from women with apple allergy and three controls were applied in a placental perfusion model with two separate cotyledons simultaneously perfused with and without apple allergen (Mal d 1). Two control placentas were perfused with compound 48/80. In outflow, histamine was quantified spectrophotofluorometrically, IL-2, IL-4, IL-6, IL-10, TNF and IFN- γ by a cytometric multiplex bead array and IL-13 and CXCL10, CXCL11, CCL17 and CCL22 with an in-house multiplex Luminex assay. Compound 48/80 induced a rapid release of histamine, CXCL10, CXCL11, CCL17 and CCL22, but not of the other factors. Apple allergen induced a time-dependent release of IL-6 and TNF, but not of histamine, in placentas of women with apple allergy compared with the unstimulated cotyledon. CCL17 levels were slightly increased after allergen stimulation in control placentas. Allergens can induce placental cytokines and chemokines distinctly in allergic and healthy mothers. These mediators may affect the prenatal development of the immune system and modify the risk of diseases related to immune disorders in childhood such as allergies.

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1. Introduction

The prevalence of allergic diseases has increased during the last few decades (Burr et al., 1989; Asher et al., 2006). Genetic factors are important for allergy development, but a time period of 30–40 years is considered to be too short for human genetic composition to undergo such dramatic changes causing this increasing prevalence. As a

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consequence, a lot of attention has been drawn to the post-natal exposure to environmental factors associated with a westernised lifestyle. Exposure to environmental factors important for allergy development appears to be important very early in life, perhaps even before birth (Jenmalm and Bjorksten, 1998). This concept was first developed in 1989, when D.J.P. Barker highlighted the possible link between events in utero and the development of diseases in adult life, called “foetal programming of diseases” (Barker et al., 1989). Prenatal farm exposure reduces the risk of asthma symptoms, allergic rhinoconjunctivitis and eczema (Douwes et al., 2008) and maternal exposure to stables during pregnancy protects against allergic sensitisation, whereas exposure later in life has limited or no effect at all (Ege et al., 2006; Lampi et al., 2011). However, the role of the gestational environment in shaping immune responses in the offspring and in the development of allergic diseases needs further investigation.

The initial exposure to allergens may occur in utero. House dust mite allergen has been detected in the amniotic fluid and in the foetal circulation, indicating a transamniotic and a transplacental transfer (Holloway et al., 2000). Dual perfusion experiments have shown a maternal–foetal passage of β -lactoglobulin, ovalbumin and birch pollen (Loibichler et al., 2002; Edelbauer et al., 2003, 2004) but also an accumulation of allergen in the syncytiotrophoblast cell layer (Szepefalusi et al., 2006). Detectable allergen-specific T cell responses at birth, shown as the capability of cord blood mononuclear cells (CBMC:s) to produce cytokines in response to allergens, support the idea of intrauterine allergen exposure and the priming of the foetal immune system (Kondo et al., 1998; Van Der Velden et al., 2001). On the other hand, the neonatal CD4⁺ T cell population has shown a typical phenotype of recent thymic emigrants, with receptors lacking the specificity of conventional T cells, and may thus be capable of interacting with a multitude of antigens, i.e. allergens (Thornton et al., 2004).

Human term placenta consists of several cell populations including fibroblasts, smooth muscle cells, endothelial cells, cyto- and syncytiotrophoblast cells and immune cells such as macrophages, T cells and mast cells. Many of these cells are able to produce cytokines and chemokines, but macrophages, endothelial cells and trophoblast cells can account for most of the production (Steinborn et al., 1998; Keelan et al., 1999). The chemokines function as attractants for leukocytes to the site of inflammation and the regulation of leucocyte maturation (Pease and Williams, 2006). The interleukin (IL)-4- and IL-13-induced chemokines CCL17 and CCL22 (Andrew et al., 1998; Nomura et al., 2002) bind to the CCR4 receptor expressed on Th2 lymphocytes, mast cells, dendritic cells and natural killer T (NKT) lymphocytes (Pease and Williams, 2006). The interferon- γ (IFN- γ)-induced chemokines CXCL10 and CXCL11 (Luster and Ravetch, 1987; Cole et al., 1998) attract CXCR3 receptor-expressing Th1 lymphocytes, NKT and mast cells (Pease and Williams, 2006).

Although allergy is associated with increased allergen-induced levels of IL-4, IL-5, IL-13, CCL17 and CCL22 by peripheral mononuclear cells (PBMCs) (Imada et al., 1995; Till et al., 1997a,b; Sun et al., 2007), little is known about the allergen-induced cytokine and chemokine production

at the local level in the placenta. Furthermore, allergen-induced mast cell degranulation in the placenta has not been demonstrated. A pronounced placental Th2 shift in allergic mothers has been suggested to explain the greater risk of maternal allergy compared with paternal allergy for the development of allergic diseases in the offspring (Ruiz et al., 1992; Liu et al., 2003). Furthermore, the higher cord blood (CB) IgE levels in children of allergic mothers than in children with paternal or no allergic history (Johnson et al., 1996; Liu et al., 2003) support a possible exaggerated placental Th2 phenotype among the allergic women. Exposure to a strong Th2 milieu during foetal development could generate long-lasting effects in the offspring by modulation of their immune responses, to an IgE favouring, Th2-like phenotype, possibly promoting allergy development later in life.

The aim of the present study was to analyse the cytokines IL-2, IL-4, IL-6, IL-10, IL-13, IFN- γ , tumour necrosis factor (TNF), the chemokines CXCL10, CXCL11, CCL17, CCL22 and histamine release in placentas after stimulation with apple allergen or the mast cell degranulating compound 48/80 in relation to maternal allergic disease.

2. Materials and methods

2.1. Subjects

Four women with an oral allergy syndrome displaying allergic symptoms to apple and 5 women without any allergic symptoms from the Jena area, region of Thuringia, Germany, were included in the study. The following inclusion criteria were applied: delivery after week 37 of pregnancy, a healthy, appropriately grown newborn, an absence of maternal chronic metabolic diseases, pharmacological therapy and pregnancy complications. In their anamneses, none of the allergic patients had reported systemic reactions, but only the classical local reactions as described for the oral allergy syndrome (Ortolani et al., 1988). The similar severity of the symptoms described did not allow subdivision of the patient group. All study participants gave their written informed consent. The regional ethics committee of the Medical Faculty of Friedrich Schiller University Jena approved the study (No. 1038-02/03).

In advance of delivery, circulating allergen-specific IgE antibodies to the major allergens of apple (Mal d 1) and birch (Bet v1; because of their cross-reactivity (Klinglmayr et al., 2009)) were measured in serum of the allergic women by using specific IgE tests (ImmunoCAP; Phadia, Freiburg, Germany) and a Phadia[®]250 system. If this was not practicable, a rapid immunographic allergy screening test (Auro Dex Visual-ENS test, including birch, other tree and grass pollen, and frequent animal allergens; Dexall, Gaithersburg, MD, USA) was conducted in the delivery room. After delivery, results were confirmed by an ImmunoCAP test. Sensitisation to additional allergens did not lead to exclusion. Both of the rapid diagnosis allergy tests were also used to exclude allergic sensitisation in the anamnestic non-allergic women. The sensitivity and specificity of the Auro-Dex Visual-Ens has been assessed previously (Pietsch, 2006).

Three of the four allergic women had in our laboratory a positive IgE test to apple or birch (CAP classes II–V) and the fourth patient showed a medical certificate for confirmation of sensitisation to apple (CAP class II). CAP classes could not be used for defining subgroups owing to the limited availability of placentas from allergic individuals. None of the non-allergic women was sensitised to the analysed allergens.

2.2. Isolation of apple allergen (Mal d 1)

Mal d 1 was extracted from fresh apples in the same lab and by using the same protocol as previously published (Rudeschko et al., 1995a,b). The concentration has been determined as described previously (Vieths et al., 1994; Rudeschko et al., 1995b). Briefly, apples (Golden Delicious from a local store) were homogenised at 4 °C in an extraction buffer containing phosphate-buffered saline (PBS; PAA, Pasching, Austria), polyvinylpyrrolidone (Sigma–Aldrich, Steinheim, Germany), ethylenediaminetetraacetic acid (EDTA; Roth, Karlsruhe, Germany), diethyl-dithiocarbamate (Sigma–Aldrich), benzamidine hydrochloride (Sigma–Aldrich) and phenylmethanesulfonyl-chloride (Sigma–Aldrich), at pH 7.4 using a pH metre (FiveEasy; Mettler-Toledo, Gießen, Germany). The homogenised apples were filtered and dialysed twice against an EDTA – diethyldithiocarbamate solution and 3 times against a tris(hydroxymethyl)aminomethane (Tris; Sigma–Aldrich) buffer, pH 8.0. The extract was applied on a Q-Sepharose Fast Flow column (Sigma–Aldrich) and eluted by the addition of Tris, pH 8.0. The total concentration of protein in the eluate was determined by a Bradford assay and the concentration of Mal d 1 with ELISA (Heinzelmann, 2005). Mal d 1 was lyophilised and stored at –20 °C. Two kilogrammes of apples generated 13 mg of Mal d 1. We have produced and used a total of two lots of the above-described allergen preparation and both have been used for perfusion of placentas of allergic and non-allergic mothers. The activity and stability of the allergen produced in this way has been reported in detail previously (Rudeschko et al., 1995a,b).

2.3. One-sided placental perfusion

Placentas were obtained after spontaneous delivery (allergic women $n=3$, non-allergic women $n=1$) or Caesarean section (allergic women $n=1$, non-allergic women $n=2$). Because of the limited accessibility to placentas from allergic women both groups have been merged. A single-sided placental perfusion system was developed that allows simultaneous separate and independent perfusion of two cotyledons of the same placenta. In all experiments one cotyledon has been perfused with apple allergen or compound 48/80 and the other with control medium. To evaluate the placental vitality and functionality in this system, we compared metabolic parameters in conditioned perfusion medium with those in dually (foetal and maternal side) perfused placentas as previously done in our laboratories (adapted according to Schneider and Huch (1985)). No significant differences in glucose

consumption, lactate production, secretion of β -hCG or consumption of oxygen were detected.

Two cotyledons from each placenta were cut out surrounded by sufficient tissue for fixation in the chambers. On the upside, the maternal tissue was penetrated by four blunt metal cannulae through the decidual plate into the intervillous space. The foetal tissue remained untouched. The perfusion medium consisted of NCTC-135 tissue culture medium (Cambrex, Verviers, Belgium) diluted 2:1 with Earl's-Buffer (Biochrom, Berlin, Germany), and supplemented with bovine serum albumin (40 g/l; MP Biomedicals, Illkirch, France), D-glucose (1.33 g/l; Merck, Darmstadt, Germany), amoxicillin (250 mg/l; Sigma–Aldrich), heparin (500 μ l/l, equivalent to 2500 IU/l, Ratiopharm, Ulm, Germany) and dextran FP40 (10 g/l, Serva, Heidelberg, Germany), adjusted to an pH of 7.4 using NaOH (Roth, Karlsruhe, Germany). For perfusion, the medium was warmed up to 37 °C and oxygenated by using a Silox-S oxygenator (Mera Senko Medical Instrument, Tokyo, Japan). The flow rate was 2.2 ml/min during the entire perfusion period. The experimental cotyledon was perfused with pure perfusion medium for 1 h followed by medium containing 4 μ g/ml Mal d 1 (similar concentrations are able to induce strong basophil activation (Erdmann et al., 2005)) or 0.1 mg/ml compound 48/80 (Sigma–Aldrich) for a further 4 to 5 h. Alternatively, after 1 h of mock perfusion, compound 48/80 was applied as a bolus of 30 mg/5 ml directly via the influx tubes into the placenta. The control cotyledon was perfused with pure perfusion medium for up to 6 h. To monitor the metabolic state, pH, pO_2 und pCO_2 were analysed every 30 min in arterial and venous flow. Venous outflow from both cotyledons was collected in 10-min steps for further analysis. To remove remaining tissue fragments, samples were centrifuged for 10 min, at 3500 $\times$ g and 4 °C. Supernatants were stored in aliquots at –20 °C until analysis.

2.4. Spectrophotofluorometric quantification of histamine

The histamine concentration in the perfusion outflow from the experimental and the control cotyledon of 2 placentas stimulated with compound 48/80 and 2 placentas stimulated with Mal d 1 was measured spectrophotofluorometrically, as described in detail elsewhere (Shore et al., 1959; Ronnberg and Hakanson, 1984). The extraction protocol and the excitation wavelengths were adapted to the analysis of perfusion medium. Histamine was extracted from 0.5 ml perfusion medium by using a mixture of 0.5 ml 0.9% NaCl (Roth), 2.5 ml n-butanol (Roth) and 0.2 ml 3 M NaOH. After a 3-min incubation on a shaker, the mixture was centrifuged at 900 g for 20 min. Two ml of the butanol phase was removed and mixed with 1.2 ml 0.12 M HCl and 3.8 ml n-heptan (Roth). After a 1-min incubation on a shaker, followed by centrifugation at 600 $\times$ g for 5 min, the n-heptan-phase was removed, cooled on ice, and mixed with 0.4 ml 0.75 M NaOH (Roth) and 0.12 ml methanolic o-phthalaldehyde (OPT; Sigma–Aldrich). After a 4-min incubation, the reaction was terminated by the addition of 0.2 ml 2 M H_3PO_4 on ice. The fluorescence was measured at $\lambda_{Ex} = 355$ nm; $\lambda_{Em} = 440$ nm using the Fluorescence HPLC Monitor RF.551 (Shimadzu, Duisburg, Germany) with 40%

MeOH/distilled water as an eluent. The fluorescence data were acquired and calculated using Chromeleon software (Dionex, Germering, Germany). The standard curve was created in duplicates, ranged from 0.5 to 50 ng/ml (dilution steps: 0; 0.5; 1; 3; 5; 10; 20; 50 ng/ml; $R^2 > 0.998$) and revealed a lower detection limit of 5 ng/ml. The coefficient of variance (CV) was below 1.5%.

2.5. Quantification of IL-2, IL-4, IL-6, IL-10, TNF, and IFN- γ using a cytometric multiplex bead array

The cytokines IL-2, IL-4, IL-6, IL-10, TNF and IFN- γ were measured in the conditioned perfusion medium by using a cytometric multiplex bead array following the manufacturer's kit instructions (Human Th1/Th2 Cytokine Kit II, BD Bioscience, Heidelberg, Germany).

Briefly, the samples (perfusion medium or standard) were mixed (1:1:1) with antibody-coated fluorescent beads ($\lambda_{Em} = 670$ nm) and the R-phycoerythrin-conjugated detection antibodies ($\lambda_{Em} = 575$ nm), and were incubated shaking for 3 h at room temperature in the dark. For the standard, recombinant proteins (IL-2, IL-4, IL-6, IL-10, TNF and IFN- γ from the assay kit) were dissolved in buffer medium and serially diluted (1:1) from 5000 to 20 pg/ml for each cytokine. The beads were washed, spun down (200 g, 5 min), and after removal of supernatants, measured on a flow cytometer (FACS Calibur; BD Bioscience) by using the implemented FCAP Array v1.0.1. kit software with the 5-parametric-curve fitting. The sensitivity limits were 2.6 pg/ml for IL-2, 2.6 pg/ml for IL-4, 3 pg/ml for IL-6, 2.8 pg/ml for IL-10, 2.8 pg/ml for TNF and 7.1 pg/ml for IFN- γ . The CV was below 10%.

2.6. Determination of CXCL10, CXCL11, CCL17, CCL22 and IL-13 by an in-house multiplex Luminex assay

The levels of CXCL10, CXCL11, CCL17, CCL22 and IL-13 in the perfusion medium were measured using an in-house multiplex Luminex assay, as described in detail elsewhere (Abrahamsson et al., 2011). Briefly, the monoclonal anti-human CXCL10 (clone 4D5, BD Biosciences, Stockholm, Sweden), CXCL11 (clone 87328, R&D Systems, Abingdon, UK), CCL17 (clone 54026, R&D Systems), CCL22 (clone 57226, R&D Systems) and IL-13 (Ref: M191302, Sanquin, Amsterdam, The Netherlands) antibodies were covalently coupled to carboxylated microspheres at a concentration of 5 μ g antibody/ 10^6 microspheres, using the protocol recommended by the manufacturer (Luminex Corporation, Austin, TX, USA). 2000 coupled microspheres were added to each well of a 1.2- μ m pore-size filter plate (Millipore multiscreen, Millipore Corporation, Bedford, MA, USA) and incubated over night with either recombinant human CXCL10, CXCL11, CCL17, CCL22 and IL-13 (R&D Systems), or samples diluted 1:2. The microspheres were washed, incubated for 1 h with biotinylated anti-human CXCL10 (1000 ng/ml, clone 6D4, BD Biosciences), CXCL11 (500 ng/ml, BAF320, R&D Systems), CCL17 (500 ng/ml, BAF364, R&D Systems), CCL22 (200 ng/ml, BAF336, R&D Systems) and IL-13 (200 ng/ml, Ref: M191304, Sanquin) antibody, followed by incubation with 1 μ g/ml Streptavidin R-phycoerythrin

conjugate (Molecular Probes, Eugene, OR, USA) for 30 min. A Luminex¹⁰⁰ instrument (Biosource, Nivelles, Belgium) was used for analysis of the samples and the data acquisition was performed using the StarStation 2.3 software (Applied Cytometry Systems, Sheffield, UK) with a 5-parametric-curve fitting. The sensitivity limits were 6 pg/ml for CXCL10 and CXCL11, 1 pg/ml for CCL17 and CCL22 and 8 pg/ml for IL-13. The samples were analysed in duplicates and the CV was below 15%. Undetectable levels were given the value of the half cut-off.

2.7. Statistical analysis

Because of the explorative nature of the study and the small sample size the data were primarily analysed by descriptive methods. To generate hypotheses about the response to allergen exposure over time mixed linear models were applied with time, allergen and their interaction as fixed factors and subject as a random factor. The level of significance was 0.05. The analyses were performed using SAS 9.3 software.

3. Results

Generally, in most cases the analytes in the perfusate decrease during the first hour of placental perfusion. The values at time point 0 represent the concentrations at the very beginning of perfusion and may be similar to serum concentrations. The first hour of perfusion is performed with pure medium without a stimulus, which usually reduces the concentrations.

3.1. Compound 48/80-induced histamine and chemokine release

To determine if mast cells in the placenta are able to degranulate, two placentas from non-allergic mothers were perfused with compound 48/80. When this was added as a bolus to the placenta 60 min after the beginning of perfusion, a strong histamine release was immediately detectable (Fig. 1). After this rapid response to compound 48/80, the histamine levels decreased to base level, followed by a second increase of histamine release after 3 h, although no further compound 48/80 was added.

The chemokines CXCL10, CXCL11, CCL17 and CCL22 (Fig. 2A–D) were also released rapidly after mast cell activation with compound 48/80, but not IL-6 (Fig. 2E) and TNF (Fig. 2F).

When 0.1 mg/ml compound 48/80 was permanently added to the perfusion medium, histamine levels were very low and only sporadically detectable during the time period analysed. In analogy to the bolus application, at the initiation of perfusion with compound 48/80, a rapid release of the chemokines CXCL10, CXCL11, CCL17 and CCL22, but not of the cytokines IL-2, IL-4, IL-13, IFN- γ , IL-6 and TNF was observed (data not shown).

3.2. Allergen-induced cytokine and chemokine release

The activity and stability of the apple allergen preparation has been demonstrated previously by performing a series of analyses including SDS-PAGE, two-dimensional

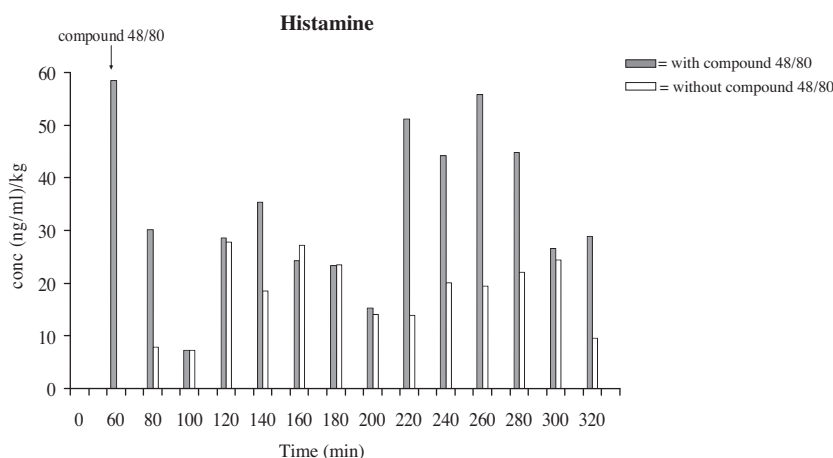


Fig. 1. Two cotyledons of a human placenta from a non-allergic woman were simultaneously and independently perfused for 320 min. After 1 h, 30 mg compound 48/80 was injected into the medium of one cotyledon (grey bars), while the other was exclusively perfused with medium as a control (white bars). The histamine concentration was analysed in the placental outflow medium in 20-min steps and has been calculated as ng histamine/ml perfusion medium/kg placenta tissue.

electrophoresis, immunoblotting, RAST inhibition, and the prick test (Rudeschko et al., 1995a,b). Perfusion of placentas with apple allergen induced a time-dependent increase of IL-6 (Fig. 3A) and TNF (Fig. 3B) compared with the unstimulated cotyledon, in placentas of women with apple allergy. The stronger increase after stimulation could be confirmed by a significant interaction between time and allergen for TNF ($F_{(1,125)} = 16.6$, $p < 0.001$) and IL-6 ($F_{(1,116)} = 25.1$, $p < 0.001$) in the mixed model analyses. IL-6 and TNF were also released spontaneously from placentas of non-allergic women, but without a further increase after the instillation of apple allergen. The CCL17 levels were slightly elevated after allergen stimulation in placentas of women without apple allergy (Fig. 3C), but the effect was not statistically significant (detailed results in Supplementary Table 1). The secreted levels of CXCL10, CXCL11 and CCL22 from the experimental and control cotyledon of the two groups were similar (data not shown), and the levels of IL-2, IL-4, IL-13 and IFN- γ were undetectable or only sporadically detectable in the samples. Histamine release was not induced by stimulation with apple allergen (all results summarised in Table 1).

Table 1

A summary of the induced analytes after compound 48/80 or Mal d 1 stimulation.

Analyte	Compound 48/80 Non-allergic, n = 2	Mal d 1 Allergic, n = 4	Mal d 1 Non-allergic, n = 3
Histamine	+	– (n = 2)	Not analysed
IL-6	–	+	–
TNF	–	+	–
CXCL10	+	–	–
CXCL11	+	–	–
CCL17	+	–	+
CCL22	+	–	–

(+) The production of the analyte was induced by stimulation with compound 48/80 or Mal d 1 compared with the unstimulated cotyledon.

(–) The production of the analyte was not induced by stimulation with compound 48/80 or Mal d 1 compared with the unstimulated cotyledon.

4. Discussion

This study has been performed to test if, in patients with an oral allergy syndrome, allergen challenge of the placenta induces release of histamine, cytokines or chemokines. As a positive control for the potential of placental mast cell reactivity, placentas have been perfused with compound 48/80. This degranulating stimulus induced histamine secretion, but no detectable effects on the cytokines analysed. On the other hand, apple allergen challenge induced secretion of IL-6 and TNF in placentas from allergic mothers. Therefore, in our system the source of both cytokines seems to be distinct from mast cells, although previous studies have demonstrated that mast cells can release TNF and IL-6 selectively upon stimulation with compound 48/80, PMA or several other stimuli, even without simultaneous histamine release (Kruger-Krasagakes et al., 1999; Gibbs et al., 2001; Kandere-Grzybowska et al., 2003; Theoharides and Kalogeromitros, 2006; Kim et al., 2007; Kulka et al., 2008), thus making it inappropriate to completely exclude the ability of mast cells in the placenta to produce these cytokines.

It may be argued that the method of delivery, with or without labour, influences cytokine levels, as reported for IL-6, but not TNF, in cord blood (Duncombe et al., 2010). The intraindividual control perfusion, one cotyledon with and one without allergen, should overcome these discrepancies at basic levels. Levels of both cytokines increase during the course of placental perfusion and can be seen to be stress markers, which have been reported in previous studies (Pierce et al., 2002; Di Santo et al., 2007). We observed this increase in both groups of allergic and healthy individuals, but the further increase in these two proinflammatory cytokines in response to apple allergen in placentas of allergic mothers indicates an enhanced general inflammatory activity in this group. Inhalant allergens have been shown to induce IL-6 and TNF production in airway epithelial cells (Vroling et al., 2007), alveolar macrophages (Chen et al., 2003), monocytes and

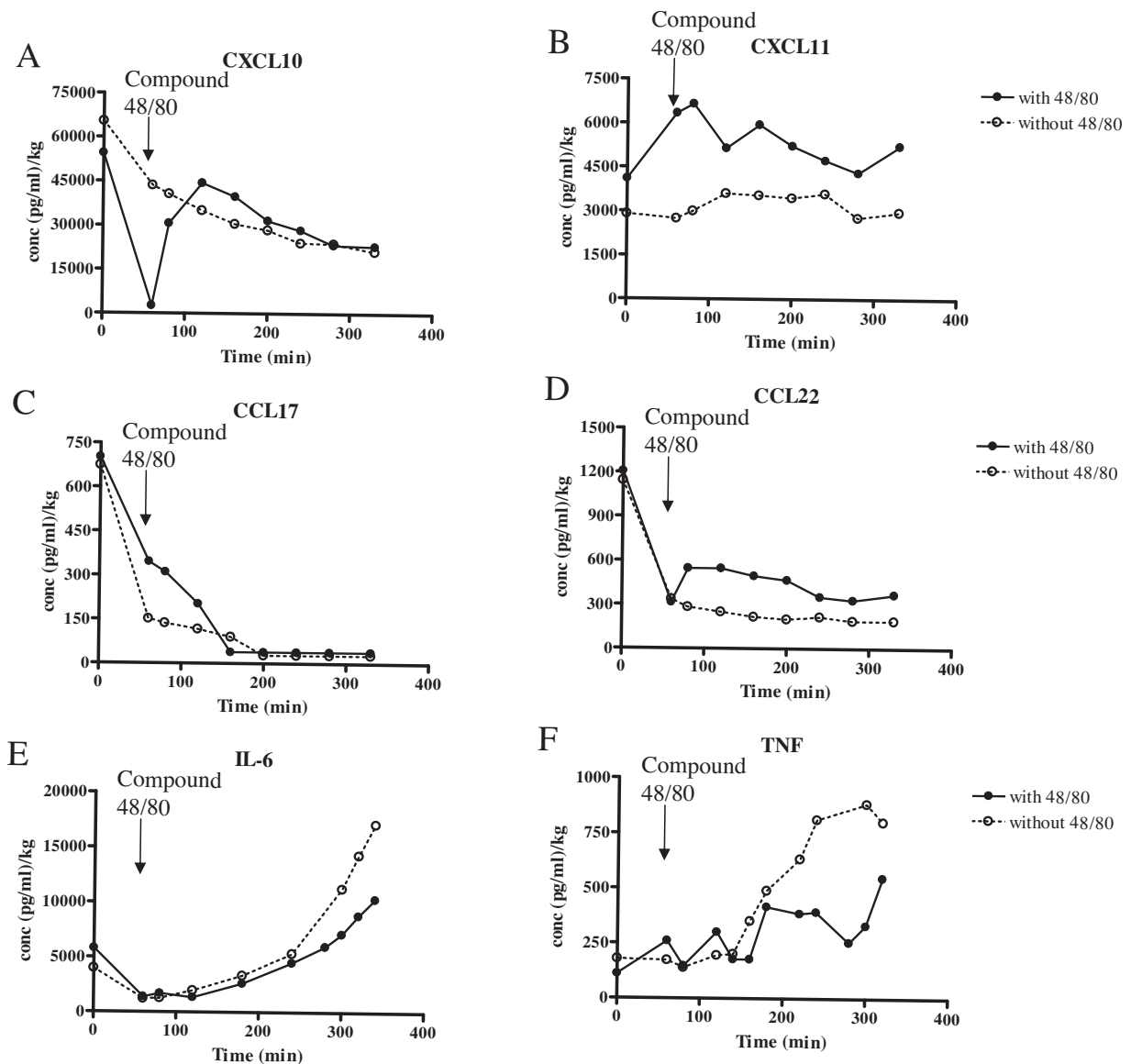


Fig. 2. Two cotyledons of a human placenta from a non-allergic woman were simultaneously and independently perfused for 320 min. After 1 h, 30 mg of compound 48/80 was injected into the medium of one cotyledon (permanent line), while the other was exclusively perfused with medium as a control (broken line). Chemokine (A, CXCL10; B, CXCL11; C, CCL17; D, CCL22) and cytokine (E, IL-6; F, TNF) concentrations were quantified by cytometric bead arrays in the placental outflow medium at several time points (dots) and calculated as pg chemokine or cytokine/ml perfusion medium/kg placenta tissue.

monocyte-derived macrophages (Andersson Lundell et al., 2005), indicating that allergens are able to evoke proinflammatory immune responses as an early response to the allergen. The allergen-induced IL-6 and TNF levels from monocytes were independent of LPS contamination, evaluated by adding the LPS-neutralising agent polymyxin B (Andersson Lundell et al., 2005). Placental perfusion systems are not sterile and although all parts of the system (e.g. tubing) are intensively cleaned and disinfected after each use, potential endotoxin contamination cannot be excluded. Nevertheless, the different responses to allergens (from the same batches) in allergic and non-allergic women, when comparing the allergen-perfused and control cotyledons, suggest that the induction of IL-6 and

TNF might be in response to the allergen rather than to endotoxins.

Even though IL-6 and TNF are not generally considered strong inducers of Th2-associated immune responses, house dust mite-stimulated alveolar macrophages from mice promote T cell proliferation and Th2-cell development by up-regulation of costimulatory B7 molecules and secretion of IL-6 and TNF, indicating a possible role for these proinflammatory cytokines in allergic inflammation (Chen et al., 2003).

Maternal allergy did not correlate with elevated Th2-like chemokine responses to apple allergen in our model, but a diminutive allergen-induced increase in CCL17 levels was observed in placentas of non-allergic women.

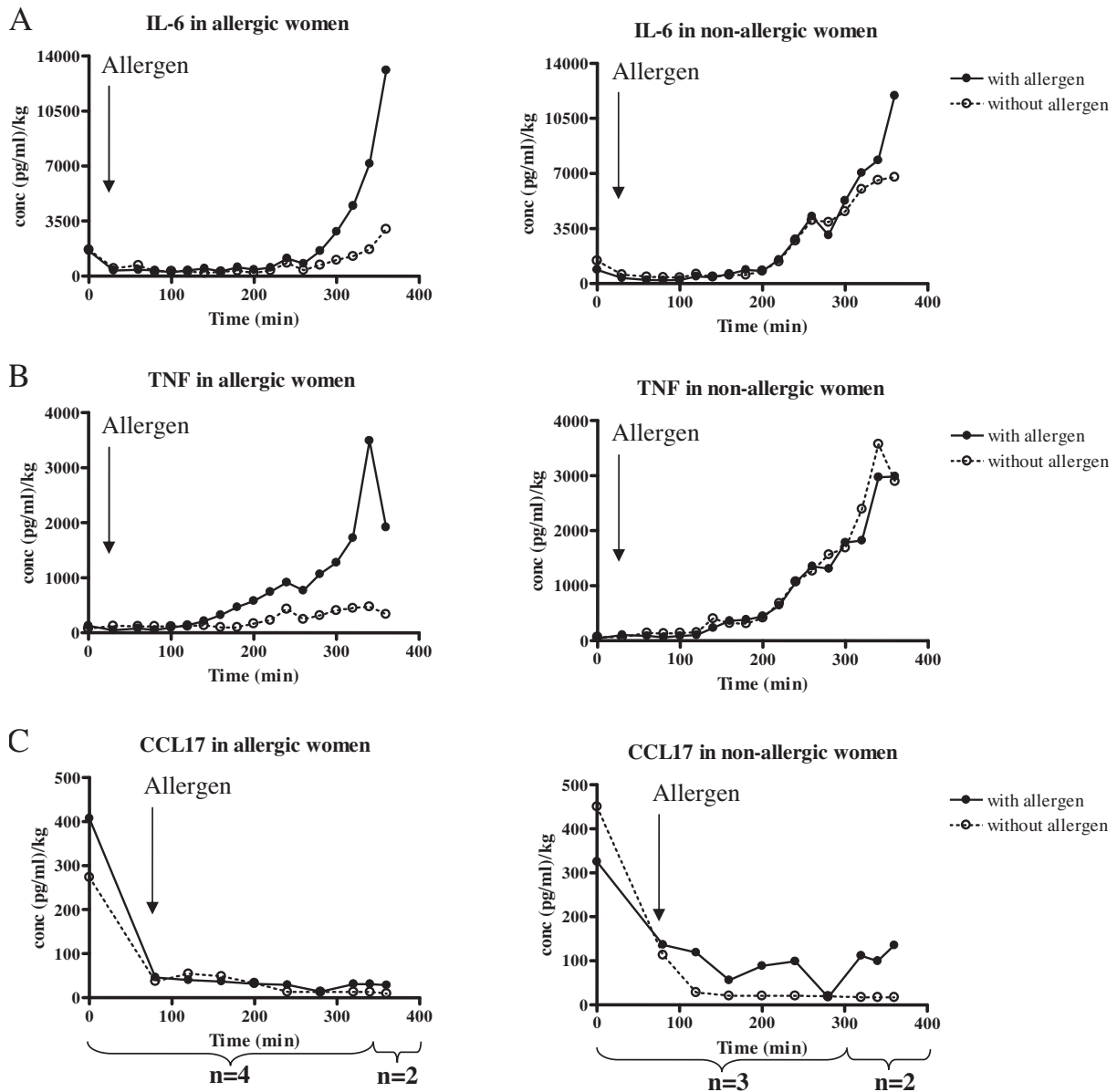


Fig. 3. Two cotyledons of human placentas from women with and without apple allergy were simultaneously and independently perfused for up to 360 min. Beginning after 1 h, one cotyledon was perfused with medium containing 4 $\mu\text{g/ml}$ Mal d 1 apple allergen (permanent line), while the other was exclusively perfused in medium as a control (broken line). IL-6, TNF and CCL17 concentrations were quantified by cytometric bead arrays in the placental outflow medium at several time points (dots) and calculated as pg/ml perfusion medium/kg placenta tissue. The spontaneous secretion of these factors during the course of perfusion has been reported previously and can be seen as the individual baseline for each placenta (Di Santo et al., 2007). For visual clarity only medians are presented (for detailed data see supplementary Table 1). By applying mixed model analyses, for TNF ($F_{(1,125)} = 16.6$, $p < 0.001$) and IL-6 ($F_{(1,116)} = 25.1$, $p < 0.001$) the stronger increase after stimulation could be confirmed by a significant interaction between time and allergen.

These findings do not necessarily exclude the possibility of enhanced Th2-like responses to allergens in the placentas of allergic women, as the signature cytokines of a Th2-like immunity, IL-4 and IL-13, were undetectable in the majority of the samples. Thus, low levels of these Th2-like cytokines might be induced, but, owing to methodological limitations, they are impossible to detect. CCL17 and CCL22 are readily detectable in the human circulation, probably explaining the high chemokine levels at the beginning (time point 0) of the perfusion experiment (Fig. 3A–D). Only

approximately 50% of the samples had detectable levels of CCL17 during the course of perfusion, which indicates that its physiological source is outside the placenta.

Mast cells contribute to the allergic inflammation by the release of granule-mediated substances such as histamine by an Fc ϵ RI-dependent pathway, whereas mast cell activation by Fc ϵ RI-independent pathways such as Toll like receptor signalling, stimulation with components from the complement system, cytokines and chemokines, may be implicated in various innate and adaptive immune

responses (Metcalfe et al., 1997; Menzies et al., 2011). The present study demonstrates the kinetics of histamine expression from placental mast cells on specific activation by compound 48/80, which indicates their potential for classical allergen-induced inflammatory reactions. A previous study has indirectly reported histamine release from placental mast cells by demonstrating a decrease in histamine in placental tissue after 90 min of perfusion with atrial natriuretic peptide (Szukiewicz et al., 2001). Degranulation of placental mast cells has also been described after a stress-induced substance P increase in murine placentas (Markert et al., 1997). Isolated uterine mast cells secrete histamine through the FcεRI-dependent pathway in response to anti-IgE stimulation (Massey et al., 1991). IgE is present in the human placenta: in the maternal as well as in the foetal tissue in women with, but also those without, allergies (Rindsjo et al., 2010). The placenta IgE levels correlate with those in blood (Joerink et al., 2009). Therefore, we did not reproduce these experiments, as all patients in our analysis had IgE to Mal d 1 and Bet v 1 in their serum. Histamine is an important mediator in the course of pregnancy, in particular during labour as it induces contractions of the myometrium, both directly and indirectly by inducing prostaglandin production, indicating that mast cell degranulation in the reproductive tract needs to be strictly regulated (reviewed in Menzies et al. (2011)). In placental perfusion system applied, histamine release was not induced by tissue perfusion with apple allergen. It may be argued that too little bioactive apple allergen concentrations have reached the mast cells. This may be because of dilution of allergens or filter effects of tissue barriers, but also influences on the stability of apple allergen such as medium or tissue components (Rudeschko et al., 1995b). As allergens may appear via the circulation in the placenta, it is tempting to speculate that the presence of a high proportion of mast cells with allergen-specific IgE antibodies attached to FcεRI in the placenta may confer a risk of preterm labour. Maternal allergy has been associated with longer gestational age, higher birth weight (Somoskovi et al., 2007) and fewer pre-term births (Savilahti et al., 2004), indicating favourable effects on the maintenance of pregnancy rather than detrimental effects, but the role of histamine in the underlying mechanisms is not known. The presence of maternal IgE in the placenta has been summarised recently. It was mainly detected around foetal Hofbauer cells in the villi, but little is known about its binding on mast cells (Rindsjo et al., 2010).

The IgE- and FcεRI-independent mechanism for mast cell degranulation by compound 48/80 is not determined, but an effect on the plasma membrane has been suggested, for example through interactions with different types of receptors, membrane transporters and translocation across the membrane (Ferry et al., 2002). Subsequent signalling through the G protein coupled receptors Mas related gene X1 (MrgX1) and MrgX2 have been suggested (Tatemoto et al., 2006; Kashem et al., 2011). We could demonstrate that mast cells in the placenta are able to synthesise and secrete chemokines upon activation, as compound 48/80 induced release of CXCL10, CXCL11, CCL17 and CCL22 in the present study. Cytokines and chemokines are in general considered to be synthesised de novo upon mast cell

activation (Kalesnikoff and Galli, 2008; Menzies et al., 2011). On the other hand, mast cells and basophils can release preformed, as well as newly synthesised, TNF (Gordon and Galli, 1991; Gibbs et al., 2001; Kulka et al., 2008), IL-4 (Gibbs et al., 1996), IL-6 (Kruger-Krasagakes et al., 1999; McCall-Culbreath, 2011) and CXCL8 (Gibbs et al., 2001) following activation. The chemokines CXCL10, CCL17, CXCL11 and CCL22 were released rapidly, only 20 min after the addition of compound 48/80. The latter two remained steadily secreted during the entire perfusion experiment (Fig. 3B and D), whereas after approximately 3 h, the concentrations of CXCL10 (Fig. 3A) and CCL17 (Fig. 3C) have decreased to levels similar to those of the control cotyledon. Thus, our data possibly indicate a rapid release of preformed chemokines, followed by a continuous release of newly synthesised CXCL11 and CCL22.

In conclusion, allergen-induced mast cell degranulation, cytokine and chemokine responses may occur in the placenta. As previously summarised, these reactions can shape or prime infant immune development. The increased allergen induced IL-6 and TNF levels in placentas of allergic women compared with non-allergic women, indicate enhanced proinflammatory immune responses to apple allergen in the allergic group, potentially influencing the shaping of immune responses in the offspring. This observation may contribute to explaining the elevated risk to newborns from allergic mothers under allergen exposure of developing allergies in later life.

Conflict of interest

None of the authors of this manuscript declares any conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jri.2014.05.001>.

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